



Improvement of shoot-tip culture proliferation in banana using PEG6000

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Abstract

In order to improve the shoot proliferation rate of 'Dwarf Cavendish' banana shoot tip cultures, polyethylene glycol (PEG₆₀₀₀) was employed at concentrations of 2, 4, and 6% (w/v). The medium without PEG was considered as negative control. An increasing of shoot proliferation was achieved in MS medium containing 2% PEG. Furthermore, application of 2% PEG significantly increased the shoot dry weight (DW) as well as root/shoot DW ratio of the regenerated explants. The root/shoot DW ratio, chlorophyll a and chlorophyll b contents of leaves were decreased by the increase in PEG percentage. Application of PEG at high concentrations increased the leaf tissue chlorosis and necrosis. The proline and potassium contents of leaf tissue were increased in regenerated shoots from the media containing 2 and 4% PEG. Application of 2% and 4% PEG increased *ex vitro* plantlets survival rate up to 34% and 40%, respectively compared to the control. In conclusion, the mild osmotic stress caused by the application of PEG 2% improved the *in vitro* shoot proliferation rate accompanied by an increase in the *ex vitro* survival rate of regenerated plantlets. Seemingly, the promising results using PEG₆₀₀₀ at the resultant level (2%) are in the main part due to the stress simulation effects and later plantlets' acclimatization through physio-biochemical mechanisms.

Keywords: *Musa spp*; osmotic stress; tissue culture; proline

Abbreviations: Chl: Chlorophyll; DW: Dry Weight; FW: Fresh Weight; PEG: Polyethylene Glycol; RWC : Relative Water Content ; TW :Turgor weight.

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Introduction

Banana is growing worldwide in more than 100 countries throughout the tropic and sub-tropic regions of the world. Nowadays, the global markets are dominated by 'Dwarf Cavendish'

cultivar due to its public favorite, transport feasibility, and unripe picking up (INIBAP, 2013). This cultivar is triploid and cannot produce fertile gametes. Therefore, the plants are generally propagated vegetatively via the asexual procedures. But, the limited number of propagules from a maternal donor, the long period of propagation cycle, serious damages of

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individual plant by the diseases and the non-homogenous growth of propagated plants are the main disadvantages of the traditional asexual propagation of banana. Currently, tissue culture techniques are routinely used in banana propagation. Continuous efforts have been made to improve the efficiency of the propagation through improvements in the culture media compositions. Ngomuo et al. (2014) reviewed the *in vitro* propagation techniques for producing banana using shoot tip cultures. Hussein (2012) studied the effects of nutrient media constituents on growth and development of banana (*Musa spp.*) shoot tips cultured *in vitro*. He found that BA concentration at 7.0 mg/l significantly promoted bud proliferation. In the same manner, Kacar et al. (2010) investigated the effects of different culture vessels and gelling agents on the multiplication of banana explants. However, debate continues about the best strategies for improving the efficiency of the tissue culture propagation methods for this highly valued crop.

Polyethylene glycol has been commonly used in plant tissue culture to induce controlled drought/osmotic stress because, it modifies the osmotic potential of the medium and induces plant water deficit in a relatively controlled manner (Bidabadi et al., 2012 a; Putnik-Delic et al., 2013). In an attempt to develop tolerant lines of banana for water stress, shoot tips were subjected to a stepwise selection procedure contained different levels (10, 20 and 30 gL⁻¹) of PEG-induced water stress (Bidabadi et al., 2012 b). They showed that water stress adversely affected fresh weight increase, shoot vigor and multiplication rate. However, a banana line L2-5 on the medium fortified with 30 gL⁻¹ PEG, had significantly the greatest fresh weight increase. The culture of tomato callus under conditions of water stress, which was induced by addition of PEG₆₀₀₀ in the medium, decreased relative growth rate and increased dry matter content in all treatments compared with the control (Aazami et al., 2010). Also, the study showed that in all studied cultivars, proline levels increased in response to water stress. Callus induction, proliferation and plantlet regeneration from the unexpanded spindle leaf sheath explants were declined with increasing the PEG level (Begum et al., 2011). Therefore, in this study we investigated the effects of PEG₆₀₀₀-

induced osmotic stress on shoot tip proliferation of *Musa spp.* cv. Dwarf Cavendish. We also examined PEG-induced acclimatization effects on *ex vitro* survival rate of regenerated plantlets.

Materials and Methods

Plantlets of banana, *Musa spp.* cv. Dwarf Cavendish, under *in vitro* culture condition were provided by Hessami Institute of Tissue Culture (Karaj, Iran) and transferred to the Horticultural Biotechnology Lab in the University of Zanjan. Twelve shoot tips (about 14 mm in length) were re-cultured in solid MS medium (Murashige and Skoog, 1962) aseptically. Micropropagation medium consisted of the MS basal medium containing 7.4 g L⁻¹ Na₂EDTA (Merck), 5.57 g L⁻¹ Fe₂SO₄ (Merck), 30 g L⁻¹ sucrose (Merck), and 6.5 g L⁻¹ agar (Sigma). The medium was supplemented with 5 mg L⁻¹ BA, 1 mg L⁻¹ IAA, 20 mg L⁻¹ glycine, 5 mg L⁻¹ nicotinic acid, 5 mg L⁻¹ pyridoxal hydrochloride, 1 mg L⁻¹ thiamin, and 100 mg L⁻¹ myo-inositol. The final pH of the media was adjusted to 5.7 with 0.1 N NaOH, and then autoclaved at 121 °C for 20 min. Osmotic stress in the reference medium was induced by adding 2, 4 and 6% (w/v) of PEG₆₀₀₀ resulting in 0.04, -0.08 and -0.12 MPa osmotic potential in the media, respectively. The medium without PEG was considered as negative control. Three explants were transferred to each experimental unit. The cultures were maintained at 24° C under 16 h illuminations. After eight weeks of explants growth, the proliferation rate (No. of shoots per explants), root and shoot dry weight (DW) and shoot DW/FW (fresh weight) ratio, No. of leaf abscission and shoots length (cm) were measured. Also, different physiological parameters including RWC, free proline, chlorophyll a (chl_a), chlorophyll b (chl_b) and potassium content in the leaf tissue were analyzed. Leaf chlorosis and necrosis were visually assessed. Measurement of the RWC was performed according to Bidabadi et al., (2012 a), and was calculated using Equation 1.

Equation (1):

$$\text{RWC(\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DT}} \times 100$$

where, FW, DW and TW represented fresh, dry, and turgor weights of leaf tissue, respectively.

Proline concentration of leaves was spectrophotometrically measured as described by Bates (1973) at 520nm using spectrometer (Milton ROY-21D). Free proline concentration was calculated in $\mu\text{mol}^{-1}\text{gr FW}$ using proline standard solution according to equation 2.

Equation (2):

$$\text{Proline}(\mu_{\text{mol}} \cdot \text{grFW}) = \frac{[\text{Proline}(\mu_{\text{gr}} \cdot \text{ml}) \times \text{Toluene}(\text{ml})] \times 155.5}{\text{SampleFW}(\text{gr})}$$

Fresh leaf samples were removed from *in vitro* regenerating shoot tips for chlorophyll measurement. The chlorophyll pigments were extracted with 90% acetone and later were assayed for their content spectrophotometrically (spectrometer model Milton ROY-21D), and calculated using Equation 3 and 4 given by Porra (2002).

Equation (3):

$$\text{Chl}_a \text{mg} \cdot \text{g}^{-1}\text{FW} = [12.7(A_{663}) - 2.69(A_{645})] \times (V/1000) \times W$$

Equation (4)

$$\text{Chl}_b \text{mg} \cdot \text{g}^{-1}\text{FW} = [22.9(A_{645}) - 4.68(A_{663})] \times (V/1000) \times W$$

in which, V is extract volume (ml), W; leaf sample weight (g), FW; leaf fresh weight (g); A_{663} ; light absorbance at 663 nm and A_{645} ; light absorbance at 645 nm. Leaf potassium content was measured based on standard potassium solution using flame photometer instrument (Jeneway PFP7-England).

To measure the survival rate of regenerated explants, the plantlets were transplanted into plastic pots containing a mixture of perlite and sand (1:2 V/V) in the greenhouse. Explants regenerated from the shoot tips cultures of 6% PEG were not transplanted successfully because of the severity of root and shoot necrosis. The transplant rate was evaluated by the number of survived plants of each treatment after four weeks growing in 75% relative humidity and 25 ± 1 °C. The experiment was conducted as completely randomized design with four replications. Analysis of variance was carried out by SAS.9.0 and means were compared using Duncan's multiple range test at 1% probability level.

Results

Application of PEG in high concentrations, especially 6% PEG caused severe leaf tissue chlorosis and necrosis (Fig. I). The proliferation rate was suppressed up to 90% using 6% PEG compared with control. Although explants showed good appearance in control medium however, high number of explants per shoot tip culture was achieved in 2% PEG and the proliferation was decreased by increasing of PEG towards (Fig. II). Based on the results, mild osmotic stress induced by 2% PEG had promotive effect on shoot proliferation rate. Using 2% PEG in shoot tip culture medium increased the shoot DW/FW as well as root/shoot DW ratios of regenerated plantlets significantly. However, these ratios were declined by any increase in the PEG concentration (Table 1). The length and RWC of regenerated plantlets were decreased by increasing the PEG concentration. Two percent PEG didn't affect the content of chl a in explants compared with control. However, the chl b content was increased by 2% PEG (Table 1).

The proline content in leaf tissue of regenerated shoots was increased by PEG treatments and the maximum amount was achieved using 4% PEG (Fig. 3 A). In this study, the highest K^+ content was achieved by 2% PEG (Fig. III B).

Discussion

Bidabadi et al. (2012a), reported the decreasing of shoot tip proliferation rate induced by the increasing of PEG in *Musa acuminata* cv. Berangan. Interestingly, our results showed contradictory findings about proliferation rate in medium containing 2% of PEG which were higher, even those than control medium. These contradictory finding could be mainly in part due to the genetically differences among banana cultivars. It is encouraging to compare these results with that were found by Benderradji, et al. (2012). They showed that moderate heat stress (30° C) increased the embryonic capacity of wheat callus in cv. Mahon-Demias. However, the heat stress with its all levels decreased the embryonic callus efficiency in cv. Hidhab. To screen the *Musa* biodiversity for drought tolerant varieties,

Vanhove et al. (2012), developed a screening test for in vitro plants. The 'ABB' variety showed the smallest stress induced growth reduction in comparison to other varieties.

Extended root development and raised root/shoot dry weight ratio are the at-hand mechanisms of plants to cope with the drought stress (Mohammadzade, Z. and Soltani, 2015). Our finding is in agreement with Putink-Delic, et al. (2013) findings whom showed the reduction of

fresh weight, tissue water content and axillary bud formation in sugar beet explants because of drought stress imposed by increasing of PEG to 5% (w/v). Both chl_a and chl_b contents were decreased by increasing the PEG concentration. These results are consistent with those of Bidabadi et al. (2012 a), who reported the decrease in chl_a and chl_b contents in regenerated plantlets of banana by increasing the PEG concentration. The chl_b hold a pivotal role in plant photosynthesis system. In

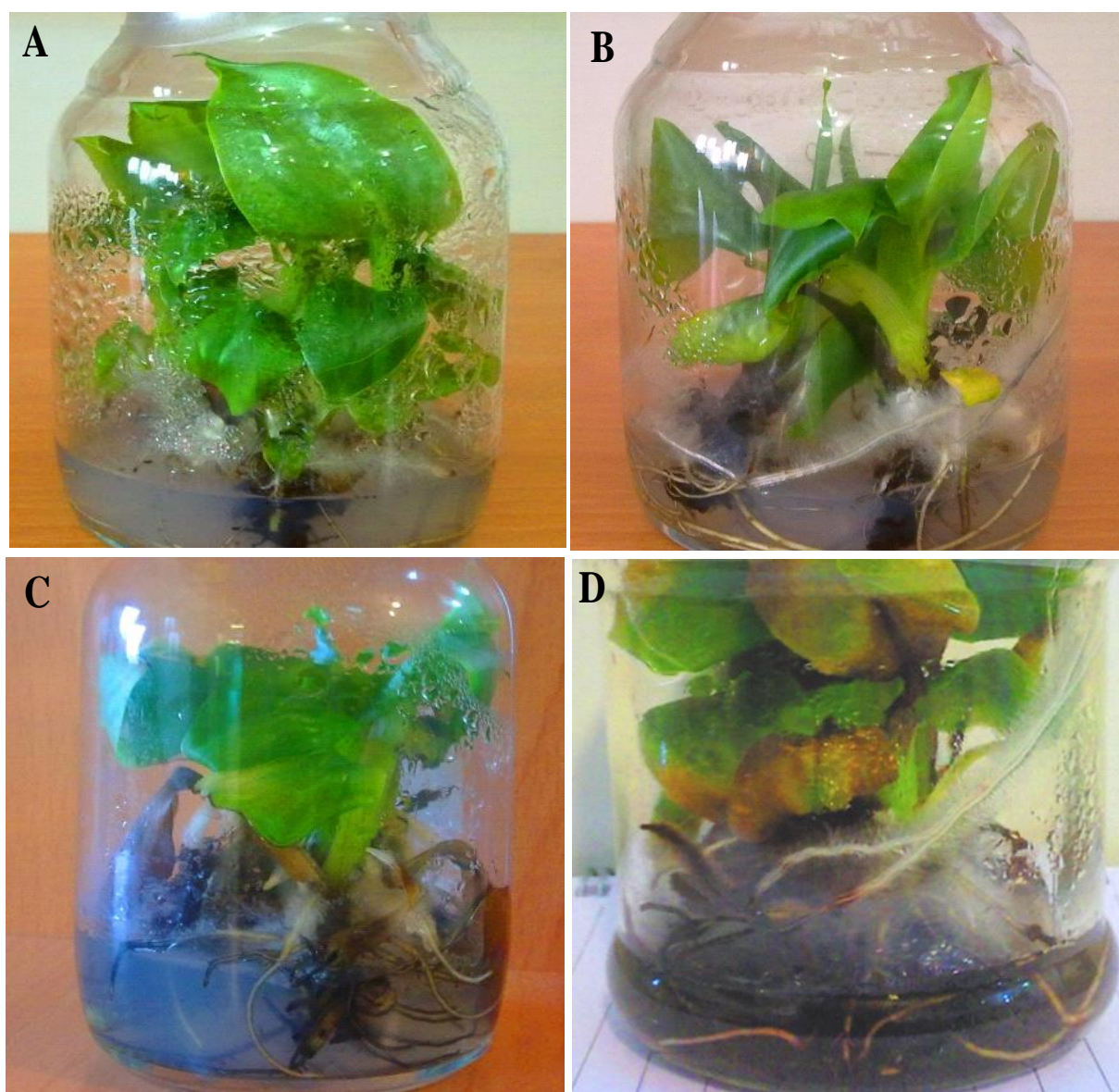


Fig. 1. Shoot proliferation, chlorosis and necrosis of 'Dwarf Cavendish' banana leaves growing at MS medium containing A (0%), B (2%), C (4%) and D (6%) of PEG₆₀₀₀.

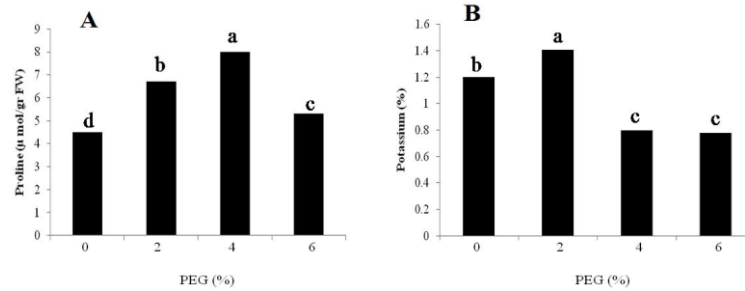


Fig. III. Mean comparison of leaf Proline (A) and Potassium (B) contents of Dwarf Cavendish' banana plantlets growing at different concentrations of PEG6000 in MS culture medium

†Means with the same letter in each column had not significantly different at 1% probability

current work, the regenerated shoots from 2% PEG treatment were retained their common photosynthesis activity by the maintenance of appropriate chl_a level as well as by the increasing chl_b levels in the leaves.

The proline content in leaf tissue of regenerated shoots was increased by PEG treatments. These results corroborated those of Putink-Delic, *et al.* (2013), who reported the increasing of leaf proline content of sugar beet explants by PEG. Bidabadi *et al.* (2012 a), reported the increase in proline content of banana explants resulted by the application of 3% PEG in culture medium. The level of proline which accumulates varies greatly and is highly dependent on the plant species (Burritt, 2012). Proline is considered to act as an osmolyte, a ROS scavenger, and a molecular chaperone stabilizing the structure of proteins, thereby protecting cells from damage caused by stress (Krasensky and Jonak, 2012).

Potassium has an important role in controlling transpiration levels and also it causes the continuity of water and other nutrient absorption through root system (Wang *et al.*, 2013). The potassium content of banana explant at 4% PEG was less than those of growing at 2% PEG. This result may be explained by the fact that 2% PEG resulted in a mild osmotic stress in comparison with its higher levels. Based on this, the plantlets were able to adjust their tissue osmotic potential to maintain the optimum physiological activities by increasing the potassium absorption.

The 2 and 4% PEG in banana shoot tip culture medium were increased the *ex vitro* plantlets survival rate up to 34% and 40%,

respectively. However, 4% PEG had no positive effect on the majority of plantlet growth and physiological traits particularly shoot proliferation rate. In sugarcane tissue culture for as an example, callus induction, proliferation and plantlet regeneration was reduced with the increased levels of PEG. However, the *ex vitro* survival rate of regenerated plantlets was increased due to their more likely drought tolerance (Begum *et al.*, 2011). The shoot and root systems of plants produced *in vitro* are fragile and vulnerable. Therefore, the transfer of plantlets from the *in vitro* to the ambient environment can be a challenge that may result in death or damage to a large percentage of micropropagated plants (Preece, 2010). Huang *et al.* (2011), studied the effects of acclimatization on *Aechmea fasciata* plantlet growth. They showed that those plantlets grown under a middle light intensity ($50 \mu\text{mol.m}^{-2}\text{s}^{-1}$) showed a dramatic increase in survival percentage and the maximum number of newly developing roots. In the case of banana, *in vitro* plantlets acclimatization caused by mild osmotic stress using 2% PEG, which affected the *ex vitro* plantlets survival by increasing their

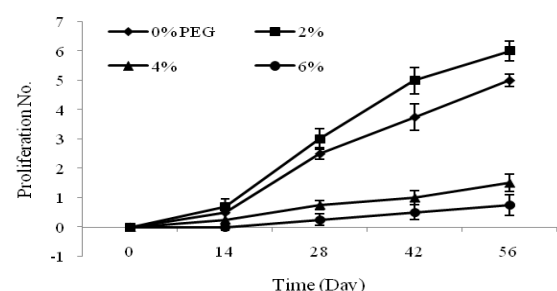


Fig. II. Trend monitoring of PEG6000 effect on proliferation No. of 'Dwarf Cavendish' banana shoot tip culture

root/shoot dry weight ratio as well as high proline and potassium contents of leaves.

As a conclusion, the mild osmotic stress caused by the application of 2% PEG in 'Dwarf Cavendish' banana tissue culture medium showed stimulatory effect on the adventitious shoot tip proliferation. It also increased the *ex vitro* survival rate of regenerated plants by influencing on their acclimatization process through growth and physiological mechanisms.

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